(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2001 (15.02.2001)

PCT

(10) International Publication Number
WO 01/11036 A1

(51) International Patent Classification[7]: C12N 15/05, 15/09, 15/29, 15/31, 15/82, A01H 5/00, 5/10

(21) International Application Number: PCT/US00/21624

(22) International Filing Date: 7 August 2000 (07.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/147,613 6 August 1999 (06.08.1999) US

(71) Applicant: THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION [US/US]; 1960 Kenny Road, Columbus, OH 43210 (US).

(72) Inventor: BISARO, David; 5815 Shannon Place Lane, Dublin, OH 43016 (US).

(74) Agent: DOCHERTY, Pamela, A.; Calfee, Halter & Griswold LLP, Suite 1400, 800 Superior Avenue, Cleveland, OH 44114 (US).

(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/11036 A1

(54) Title: METHOD OF ENHANCING PLANT RESISTANCE TO PATHOGENS

(57) Abstract: A method of preparing plants with enhanced resistance to infection with plant pathogens is provided. The method comprises transforming a plant cell with a DNA construct which comprises an exogenous SNF-1 transgene, i.e., a DNA which encodes an SNF-1 protein kinase or the catalytic domain of such kinase. The transgene also comprises a promoter which regulates expression of the SNF-1 kinase or the catalytic domain. The promoter is operably linked to the DNA sequence which encodes the SNF-1 kinase or catalytic domain. The method further comprises the step of generating a transformed plant from the transformed plant cell. The transformed plant expresses the SNF-1 kinase or the catalytic domain and, thus, contains an SNF-1 kinase or catalytic domain that is encoded by the SNF-1 transgene as well as the SNF-1 kinase that is encoded by the plants own SNF-1 gene. Also provided is a plant cell having a SNF-1 transgene stably integrated into its genome. The transgene comprises a DNA sequence encoding a SNF-1 kinase or the catalytic domain of such kinase and a promoter which controls expression of the DNA coding sequence in the plant cell. The present invention also relates to plants regenerated from such transformed cells and seeds of such transformed plants.

METHOD OF ENHANCING PLANT RESISTANCE TO PATHOGENS

CROSS-REFERENCE TO RELATED APPLICATION

Under 35 USC §119(e)(1), this application claims the benefit of prior U.S. provisional application 60/147,613, filed August 6, 1999.

BACKGROUND

Plant pathogens are of great economic importance, as plant disease accounts for a significant fraction of crop losses. The present invention provides a method of making plants with enhanced resistance to infection with plant pathogens, including viral pathogens, bacterial pathogens, and fungal pathogens.

SUMMARY OF THE INVENTION

The present invention provides a method of preparing plants with enhanced resistance to infection with plant pathogens. The method comprises transforming a plant cell with a DNA construct which comprises an exogenous SNF-1 transgene, i.e., a DNA which encodes an SNF-1 protein kinase or the catalytic domain of such kinase. The transgene also comprises a promoter which regulates expression of the SNF-1 kinase or the catalytic domain. The promoter is operably linked to the DNA sequence which encodes the SNF-1 kinase or catalytic domain. The method further comprises the step of generating a transformed plant from the transformed plant cell. The transformed plant expresses the SNF-1 kinase or the catalytic domain and, thus, contains an SNF-1 kinase or catalytic domain that is encoded by the SNF-1 transgene as well as the SNF-1 kinase that is encoded by the plants own SNF-1

gene. Such plants are referred to as "overexpressors." The present method is especially useful for producing plants with enhanced resistance to plant pathogens, particularly viral pathogens, more particularly Geminiviruses. It is expected that the present method is also useful for producing plants with enhanced resistance to abiotic stress. Examples of abiotic stress are ozone, heat stress, and salt stress.

The present invention also provides a plant cell having a SNF-1 transgene stably integrated into its genome. The transgene comprises a DNA sequence encoding a SNF-1 kinase or the catalytic domain of such kinase and a promoter which controls expression of the DNA coding sequence in the plant cell. The present invention also relates to cell cultures consisting of such transformed cells, plants regenerated from such transformed cells and seeds of such transformed plants.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence, SEQ ID NO: 1, and amino acid sequence, SEQ ID NO:2, of SNF1 kinase from *Arabidopsis thaliana*. The cDNA was obtained from a two-hybrid screen and sequenced by standard methods. The sequence is identical to a previously reported SNF1 cDNA from the same species (Le Guen, L., Thomas, M., Bianchi, M., Halford, N.G., and Kreis, M. (1992) Structure and expression of a gene from *Arabidopsis thaliana* encoding a protein related to SNF1 protein kinase. Gene 120: 249-254).

Figure 2 shows an amino acid sequence alignment of SNF1 proteins from yeast , SEQ ID NO:3, Arabidopsis, SEQ ID NO:2, and tobacco, SEQ ID NO:4. The sequences shown

were obtained from GenBank and aligned using the ClustalW algorithm.

Figure 3. Mean latent period following Beet Curly Top Virus (BCTV) inoculation of transgenic antisense SNF1 plants. Non-transgenic *Nicotania. benthamiana* plants and plants representing three independent *N. benthamiana* transgenic lines expressing an antisense SNF1 construct (AS-4, AS-5, and AS-12) were agroinoculated with a standard dose of BCTV (OD600 = 1.0). The mean latent period (days post-inoculation) is indicated, and the number of infected versus inoculated plants for each treatment is given in parenthesis. Note that the latent period for BCTV on non-transgenic plants is approximately 21 days, whereas the latent period observed for the three transgenic lines tested in this experiment were approximately 14 days (lines AS-5 and AS-12) and 16 days (line (AS-4).

Figure 4. BCTV ID_{50} of non-transgenic and antisense SNF1 plants. Non-transgenic *N. benthamiana* plants and plants representing two independent transgenic lines expressing an antisense SNF1 construct (AS-4 and AS-12) were agroinoculated with varying doses BCTV, beginning with the standard dose (OD600 = 1.0) followed by serial 5-fold dilutions of the standard dose. The percent of plants in the sample infected at each inoculum dose was noted and plotted versus the log5 of the dilution. The data represent the average of three independent experiments, with 16 plants for each treatment in each experiment. Note that the ID_{50} for BCTV on non-transgenic plants is reached at approximately 18-fold dilution of the inoculum, whereas the ID_{50} is reached at 1,150-fold dilution in line AS-4, and following 6,250-

fold dilution in line AS-12.

Figure 5 is a graph showing BCTV ID_{50} values on non-transgenic and a sense (overexpressing) SNF1 line. Non-transgenic *N. benthamiana* plants and plants representing a transgenic line expressing a sense SNF1 construct (S-1) were agroinoculated with varying doses of BCTV, beginning with the standard dose (OD600 = 1.0) followed by serial 5-fold dilutions of the standard dose. The percent of plants in the sample infected at each inoculum dose was noted and plotted versus the log5 of the dilution. The data represent the average of four independent experiments, with 16 plants for each treatment in each experiment. Note that the ID_{50} for BCTV on non-transgenic plants is reached at approximately 18-fold dilution of the inoculum, whereas an inoculum greater than the standard dose is needed to achieve the ID_{50} for line S-1

DETAILED DESCRIPTION OF THE INVENTION

The present method provides a method of transforming a plant cell which is useful for preparing a plant with enhanced resistance to plant pathogens, particularly viral pathogens, and to abiotic stress. The method of transforming the cell comprises the steps of introducing into a plant sample an exogenous DNA fragment which comprises a transgene comprising a sequence which encodes a SNF-1 kinase protein or the catalytic domain thereof and a promoter which is operably linked to SNF-1 kinase encoding sequence, i.e., the promoter controls expression of the SNF-1 kinase or catalytic domain. The cells are then grown under conditions that allow for expression of the SNF-1 kinase or SNF-1 catalytic domain, and,

preferably, expression of a selectable or screenable marker gene that, preferably, is co-introduced into the plant sample with the SNF1 transgene. The marker gene may be on the same DNA fragment as the SNF-1 transgene or different DNA fragment.

Thereafter, cells which contain and express the SNF-1 transgene are selected and used to generate pathogen resistant transgenic plants. Expression of the transgene, preferably, is assayed by conventional techniques such as for example Northern analysis or RT-PCR. The transgenic plants produced in accordance with the present method contain the transgene within the genome of their cells, i.e., the transgenic plants are stably transformed. It has been determined that such transgenic plants are resistant to infection with geminiviruses, particularly Beet Curly Top Virus (BCTV). As used herein, the term "resistant", means a significant increase in the amount of geminivirus required to produce disease symptoms as compared to a similar non-transgenic plant which does not contain the transgene. In the case of the geminivirus BCTV, which infects dicotyledonous plants in over 70 different families, symptoms include curling and deformation of new leaves at the apex followed by severe stunting. In the case of the geminivirus, Tomato Golden Mosaic Virus (TGMV), which infects dolonaceous plants such as tobacco, tomato, and pepper, such disease symptoms include curling and deformation of new leaves at the shoot apex as well as the appearance of golden mosaic (yellow) areas in the affected leaves. Alternatively, resistance is monitored by assaying virus accumulation using conventional techniques such as Southern analysis using a viral DNA probe.

SNF-1 Kinase

SNF1 is a serine/threonine kinase that plays a key role in glucose sensing and signal transduction pathways in yeast and plant cells. A similar role is ascribed to the homologous AMP-activated protein kinase (AMPK) in mammalian cells (for review see Johnston, M. (1999) Feasting, fasting, and fermenting: glucose sensing in yeast and other cells. Trends in Genetics 15: 29-33). In yeast, SNF1 kinase is required for the expression of glucose-repressed genes (e.g. *SUC2*, which encodes invertase, an enzyme that hydrolyzes sucrose to glucose and fructose). In addition to enzymes involved in carbohydrate metabolism, SNF1 kinase also regulates enzymes involved in lipid metabolism, and is also required for normal cell cycle control in yeast. Plant homologues have been cloned from *Arabidopsis*, tobacco, potato, barley, and rye. The amino acid sequences of the *Arabidopsis* and tobacco SNF-1 kinase are shown in Figure 2. The tobacco and rye SNF1 proteins have been shown to complement yeast *snf1* mutants, suggesting that the function of the SNF1 protein is conserved between yeast and plants.

SNF-1 Transgene

As used herein, a SNF-1 transgene is a polynucleotide having a sequence which encodes a protein whose amino acid sequence is at least 90% identical, preferably 95% identical, more preferably at least 97% identical to the amino acid sequence of a plant or yeast SNF-1 kinase or to the amino acid sequence of the catalytic domain of a plant or yeast SNF-1 kinase. For the SNF-1 transgenes which encode the truncated SNF-1

protein kinase, i.e., the catalytic domain, it is preferred that the coding sequence encode the N terminal portion of the plant or yeast SNF-1 kinase . For plant SNF-1 kinase, the preferred N terminal portion comprises from about amino acid 1 to about amino acid 350. For yeast SNF-1 kinase, the preferred N-terminal portion comprises from about amino acid 1 to about amino acid 400. Such N-terminal portion of the plant and yeast SNF1 proteins contains the putative ATP binding site as well as subdomains typically found in protein kinases.

The SNF-1 encoding sequence may be a heterologous SNF-1 encoding sequence, i.e., an SNF-1 gene from yeast or a different plant species. For example, a tobacco plant may be transformed with an SNF-1 gene from *Arabidopsis*. Alternatively, the encoding sequence may be a homologous SNF-1 kinase encoding sequence, i.e., an SNF-1 gene from the same plant species. For example, a tobacco plant may be transformed with an SNF-1 gene from another tobacco plant.

The protein encoded by the SNF-1 transgene need not have an amino acid sequence which is 100% identical to a known amino acid sequence, referred to hereinafter as a "reference sequence". Such protein may have an altered sequence in which one or more of the amino acids in the reference sequence is deleted or substituted, or one or more amino acids are inserted into the sequence of the reference amino acid sequence. As a result of the alterations, the altered protein has an amino acid sequence which is at least 90% identical to the reference sequence, preferably at least 95% identical, more preferably at least 97% identical, most preferably at least 99% identical to the reference sequence. Altered sequences which are at least 95% identical have no more

than 5 alterations, i.e. any combination of deletions, insertions or substitutions, per 100 amino acids of the reference sequence. Percent identity is determined by comparing the amino acid sequence of the variant with the reference sequence using MEGALIGN project in the DNA STAR program. Sequences are aligned for identity calculations using the method of the software basic local alignment search tool in the BLAST network service (the National Center for Biotechnology Information, Bethesda, MD) which employs the method of Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403-410. Identities are calculated by the Align program (DNAstar, Inc.) In all cases, internal gaps and amino acid insertions in the candidate sequence as aligned are not ignored when making the identity calculation. The alterations are designed not to abolish the kinase activity of the altered protein or polypeptide.

While it is possible to have nonconservative amino acid substitutions, it is preferred that the substitutions be conservative amino acid substitutions, in which the substituted amino acid has similar structural or chemical properties with the corresponding amino acid in the reference sequence. By way of example, conservative amino acid substitutions involve substitution of one aliphatic or hydrophobic amino acids, e.g. alanine, valine, leucine and isoleucine, with another; substitution of one hydroxyl-containing amino acid, e.g. serine and threonine, with another; substitution of one acidic residue, e.g. glutamic acid or aspartic acid, with another; replacement of one amide-containing residue, e.g. asparagine and glutamine, with another; replacement of one aromatic residue, e.g.

phenylalanine and tyrosine, with another; replacement of one basic residue, e.g. lysine, arginine and histidine, with another; and replacement of one small amino acid, e.g., alanine, serine, threonine, methionine, and glycine, with another.

The transgene further comprises a promoter which is operably linked to the SNF-1 coding sequence for expression of the coding sequence. Preferably, the transgene further comprises a polyadenylation signal. The promoter, preferably, is a plant promoter, for example the 35S cauliflower mosaic virus (CaMV) promoter or a nopaline synthase or octopine synthase promoter. Examples of other constitutive promoters used in plants are the 19 S promoter, and promoters from genes encoding actin or ubiquitin. Optionally, the promoter is a regulatable or inducible promoter. One example of an inducible promoter is the chemically inducible promoter known as the tobacco PR-1a promoter. Another example of an inducible promoter is one which is wound inducible. Such promoters are described by Stanford et al., Mol. Gen. Genet. 215: 200-208 (1989); Xu et al., Plant Molec. Biol. 22: 573-588 (1993), Logemann et al., Plant Cell 1: 151-158 (1989); Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993); Firek et al., Plant Molec. Biol. 22: 129-142 (1993); and Warner et al., Plant J. 3: 191-201 (1993). Other suitable promoters include tissue specific promoters. Examples of such promoters are green tissue specific promoters, root specific promoters, stem specific promoters, and flower specific promoters such as those described by Hudspeth & Gurla, Plant Molec. Biol. 12: 579-589 (1989) and de Framond, FEBS 290: 103-106 (1991).

For the purposes of maximizing yield in crop plants,

it may be desirable to control SNF1 expression in transgenic plants since gross overexpression of SNF1, in some cases, may prove toxic. Such control, preferably, is achieved by using promoters less active than the normally strong and constitutive 35S CaMV promoter, or by selecting 35S lines that are low level expressors. Additional control is also achieved by placing the transgene under the control of tissue specific and/or developmentally regulated promoters, or by using inducible promoters (e.g. a glucocorticoid-inducible promoter).

In addition to the transgene, the exogenous DNA fragment, preferably, also comprises other appropriate regulatory signals, such as a leader sequence, transcription terminator, and polyadenylation site , which direct expression of the operably linked SNF-1 coding sequence in the plant cell. Such regulatory signals are readily available in the art.

Plant Cell Transformation with the Transgene

Suitable plant cells are from monocotyledonous or dicotyledonous plant. Suitable monocotyledous species are, by way of example, barley, wheat, maize and rice. Suitable dicotyledonous species include, but are not limited to, tobacco, tomato, sunflower, petunia, cotton, sugarbeet, potato, lettuce, melon, soybean, canola and pepper. Thus, the method is useful for conferring enhanced pathogen resistance to a wide variety of plants. Agricultural crop plants are of particular importance. Any type or source of plant cells which serve as target for transformation by one or more delivery methods can serve as the host cells for transformation. Such sources include, by way of example, immature and mature embryos, pollen, protoplasts, suspension.

Delivery of the DNA fragment in to the host plant cells may be accomplished by a variety of techniques available in the art. Such techniques include non-biological mechanisms such as microprojectile bombardment, electroporation, microinjection, induced uptake, and aerosol beam injection.

Optionally, the DNA construct comprising the exogenous SNF-1 transgene may be subcloned into a vector effective for introducing the DNA construct into the plant. Ti plasmid vectors effective for this purpose are pMON 530, pBI221, pGMVNEO pCMC1100, and pDG208. In a preferred embodiment, the DNA construct is subcloned into a binary Ti plasmid plant vector and mobilized into *Agrobacterium. Tumefaciens*, and the *A. Tume*faciens transformant is then used for infection and transformation plant cells or tissues. Binary plant transformation vectors are known in the art

Preferably, the SNF1 gene cloned in a Ti plasmid vector is introduced into the plant sample using an *Agrobacterium* transformant. The *Agrobacterium* transformant is cocultivated with plant cells or plant tissues. The *Agrobacterium* binds to the plant cell walls and transfers the plasmid or a portion thereof into the plant cell. Where the vector is *Agrobacterium tumefaciens*, transformation results from the transfer of a specific portion of the plasmid, referred to hereinafter as "T-DNA", into the genome of plant cells. The T-DNA is transferred and integrated into the plant genome as a discrete unit. The T-DNA contains the exogenous SNF-1 gene or the catalytic domain thereof, which, preferably, is flanked by a promoter and polyadenylation signals. Preferably, the T-DNA also contains a screenable marker gene, or or selectable

marker resistance gene, such as Tn5 neomycin phosphotransferase II, which confers resistance to kanamycin.

The transformed plant cells are selected by growth in selection medium. Thereafter, transformed plants are regenerated from the cells using conventional techniques and analyzed to ensure that the transformed plant contains the exogenous gene and is expressing the exogenous gene.

Leaf discs or tissue cultures of transformed plant cells are propagated to generate transformed whole plants. The transformed leaf discs or plant cell are cultured on a suitable medium, preferably, a selectable growth medium. Plants may be regenerated from the resulting callus. Transgenic plants are those whose cells stably integrate the exogenous transgene into their genome, the exogenous gene being expressible in the cells. Resistance or sensitivity of the transgenic plant to a pathogen is assessed by the ability of the plants to grow, grow faster, or avoid disease symptoms in the presence of a predetermined dose or inoculum of the pathogen as compared to plants of the same species which have not been transformed in accordance with the present method.

Enhanced Susceptibility of Plants Expressing Antisense Arabidopsis SNF-1 Kinase

Our interest in SNF1 kinase began with the discovery that two geminiviral proteins, TrAP (AL2) from tomato golden mosaic virus, and L2 protein from beet curly top virus, interact with SNF1 kinase. We further found that transgenic *Nicotiana benthamiana* plants expressing full-length or truncated versions of the viral proteins show enhanced susceptibility (ES) to virus

infection, characterized by a decreased latent period (time to appearance of disease symptoms) and reduced ID_{50} values (inoculum dose required to infect 50% of plants in a given sample). Interestingly, these transgenic plants show ES not only to the DNA-containing geminiviruses TGMV and BCTV, but also to the RNA virus tobacco mosaic virus, indicating that the ES phenotype is quite general and may extend to all viruses, bacterial pathogens, fungal pathogens, and abiotic stress. We hypothesized that one function of the TrAP and L2 proteins during the geminiviral infection process is to inhibit the activity of SNF1 kinase, thereby disabling a general host defense. To test our hypothesis we attempted to reproduce the ES phenotype by expressing an antisense SNF1 kinase construct (driven by the CaMV 35S promoter) in transgenic plants. Transgenic *N. benthamiana* plants comprising the exogenous *Arabidopsis* SNF-1 gene in antisense orientation relative to the 35S promoter were tested by challenge inoculation with BCTV. Viruses were delivered to plants by the agroinoculation procedure described in Elmer et al. (1988) *Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAS*. Plant Mol. Biol. 10:225-234.

The data shown in Figures 3 and 4 show that the ES phenotype does in fact result following expression of antisense SNF1 kinase in transgenic *N. benthamiana* plants. The ES phenotype is characterized by a reduction in mean latent period of from 5-7 days (Fig. 3), and a reduction in viral ID_{50} from 60- to 330- fold (Fig. 4), depending on the transgenic line. Clearly, infection levels comparable to those seen with non-transgenic plants can be achieved with much less virus inoculum in the case of the transgenic antisense SNF1 lines.

Example 1

Transgenic Plants Expressing an Exogenous SNF-1 Transgene.

A. Cloning of *Arabidopsis* SNF-1 gene

The nucleotide sequence, SEQ ID NO: 1, and amino acid sequence, SEQ ID NO:2, of SNF1 kinase from *Arabidopsis thaliana* are shown in Figure 1.

The SNF1 gene was obtained in a yeast two-hybrid screen using a truncated TGMV TrAP protein as bait, and an Arabidopsis cDNA library as prey. The cDNA was full-length, and was recognized as encoding SNF1 by virtue of its homology to yeast SNF1 and to the tobacco SNF1 proteins (Fig. 2), and by its identity to previously cloned Arabidopsis SNF1 (Le Guen, L., Thomas, M., Bianchi, M., Halford, N.G., and Kreis, M. (1992) Structure and expression of a gene from Arabidopsis thaliana encoding a protein related to SNF1 protein kinase. Gene 120: 249-254). (Fig. 1 and 2).

The SNF1 gene was cloned from the yeast two-hybrid vector by PCR using GCGCTCGAGACCATGGATCATTCATCAAATAGATTTGGCAATAATGG , SEQ ID NO: 5, as the 5' primer and GCGGGATCCTCAGATCACACGAAGCTC, SEQ ID NO: 6, as 3' primer. The 5' primer adds an XhoI site to the 5' end of the SNF1 sequence, while the 3' primer adds a BamHI site. The PCR product was subsequently cleaved with XhoI and BamHI and cloned into the similarly cleaved, plasmid vector pET3 (Clonetech).

In addition to obtaining SNF1 from a two-hybrid clone as described above, it should be possible to obtain SNF1 cDNA by RT-PCR from any species in which the SNF1 sequence is known such as for example, tobacco, and

potato, barley and rye. The sequences of the known SNF1 genes are generally available on a publically available database such as GenBank.

To create recombinant Ti plasmids for agrobacterium-mediated plant transformation, the Ti plasmid vector pMON530 was cut with SmaI and treated with calf intestinal alkaline phosphatase to prevent re-ligation. The SNF1 sequence was then removed from pET by cutting with NdeI (from the pET3 polylinker) and BamHI, and rendered flush ended by treatment with T4 DNA polymerase. The flush-ended pMON530 and SNF1 DNA fragments were mixed and ligated, and used to transform *E. coli*. Clones containing the SNF1 gene in the sense and antisense orientation relative to the 35S promoter in pMON530 were selected and mated into *A. tumefaciens* for transformation of plants.

B. Materials and Methods Used to Prepare *A. tumefaciens* Transformant

The binary system consists of two components: 1) a disarmed *A. tumefaciens* Ti plasmid, which provides functions required for excision of the T-DNA from the Ti plasmid, and for its transfer and integration into the plant genome and 2) a second, smaller binary plasmid vector of about 6 - 10 kb that contains the T-DNA to be transferred, i.e. the *Arabadopsis* SNF-1 gene and a drug resistance gene.

Cultures of E. coli harboring the binary vectors were grown in LB broth containing 50 μg/ml spectinomycin. The binary vectors contained the *Arabadopsis* SNF-1 transgene and a selectable marker. Many binary vectors also include a streptomycin/spectinomycin resistance gene (outside the

T-DNA) for selection in E. coli and *A. tumefaciens*. The binary vector employed was pMON530 prepared as described in (Rogers et al. (1987) *Improved vectors for plant transformation: Expression cassette vectors and new selectable markers*. Methods in Enzymology 153: 253-277).

Non-transformed *A. tumefaciens* containing the resident, disarmed Ti plasmid was obtained from Monsanto. Cultures of the non-transformed *A. tumefaciens* were grown overnight in LB broth containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin. Cultures of E. coli containing the mobilization plasmid (e.g. pRK2013) were grown overnight in LB broth containing 50 μg/ml kanamycin. Cultures of E. coli containing the binary plasmid were grown overnight in LB broth containing 50 μg/ml spectinomycin.

1-2 drops of each overnight culture were mixed and streaked on an LB plate containing no selection marker. The plates were incubated at 28°C until colonies appeared, usually for about 6 days. Then a small amount of culture was recovered from the LB plate and streaked on LB plates containing the selection markers or antibiotics which corresponded to the resistance markers of the *A. tumefaciens* recipient containing the disarmed Ti plasmid and the binary vector to select *A. tumefaciens* transformants.

The plates were incubated at 28°C until colonies were detected on the plates.

Several individual colonies were recovered from each plate and inoculated into LB broth containing the same antibiotics as the plate and incubate at 28°C with shaking.

C. Transforming Plant Samples

Plasmid DNA was isolated from *A. tumefaciens*

transformants according to the method of Dhaese et al. (1979) Nucleic Acids Research 7: 1837 and used to verify the presence and integrity of Arabidopsis SNF-1 gene in T-DNA by Southern blot analysis or PCR.

A. tumefaciens transformants were also used to transform leaf discs *from Nicotiana benthamiana* plants. This step varies depending on the species to be transformed. For petunia and tobacco leaf discs are also used. For tomato, cotyledon pieces are used. For *Arabidopsis thaliana*, sterile root pieces are used. Prior to transformation, the plant tissues are sterilized in a solution of 20% Clorox, 0.5% Tween 20 for 15 min.

The leaf discs were placed upside down on MS104 plates and preincubated for 48 hours at room temperature in continuous light to increase the transformation efficiency Thereafter, the sterilized leaf discs were soaked in a liquid culture of an *A. tumefaciens* transformant. This is done by placing 10-20 discs in a sterilin tube (with a loose cap) and adding 1 ml of an overnight culture. The discs were then removed, blotted dry with sterile filter paper, and placed upside down on an MS104 plate with no selection. After 48 hr on the non-selective MS104 media at room temperature, the discs were transferred to MS104 plates containing selection medium (750 μg/ml carbenicillin to kill the Agrobacterium and 300 μg/ml kanamycin to select for the desired T-DNA marker). After about 1-2 weeks, the discs form callus around the edges of the disc. This is followed by the appearance of shoots.

Shoots were removed at regular intervals from the callus and transferred to rooting media (MSO plates containing the antibiotics present in the MS104 plates). Shoots with roots were transferred to sterile soil in

pots and covered with clear plastic to retain humidity. After 2-4 days, plastic can be removed and transgenic plants treated as normal plats.

Transformed leaf discs are harvested and analyzed for presence of SNF1 transgene and mRNA after 2-6 days. Alternatively transformed regenerants are obtained and analyzed in the same manner. DNA, RNA or protein is isolated from the leaf discs or regenerated plants by conventional methods. The presence of an integrated SNF-1 transgene in the genome of the plant is examined by restriction endonuclease digestion followed by Southern blot analysis, or by PCR using primers designed to recognize T-DNA border sequences or by PCR using primers designed to amplify a region within the transgene. Expression of RNA encoding the SNF-1 transgene is examined by Northern blot analysis or by RT-PCR. Expression of protein is examined by Western blotting.

Transgenic lines are established by selfing transformed plants to homozygosity using conventional techniques.

The presence of sense transgenes in plants from lines S-1, S-2, S-3, S-5, and S-6 was verified by PCR, using the following primers: GATGTATGGAGTTGCG, SEQ ID NO; 7, and CGCATAGGATTGGAACC, SEQ ID NO:8. These primers lie within the SNF1 gene itself, and they amplified a fragment of about 500 bp from plants representing each of the transgenic lines. In addition, the transgenic plants are kanamycin resistant and seeds are routinely germinated on medium containing kanamycin. Kan resistance is conferred by the Ti plasmid vector. Further, non-transgenic plants are kan sensitive, and the primers do not amplify anything when non-transgenic plant DNA is used as template for PCR.

Northern blot analysis, using as probe band isolated and random primer labeled *Arabidopsis* SNF1 gene, showed a transcript of about 2 kb in RNA isolated from antisense and sense transgenic plants of all lines. The blots were done under high stringency conditions, so a signal from the endogenous tobacco SNF1 transcript(s) was not seen. There was no signal in
RNA from control, non-transgenic plants.

D. Enhanced Resistance of Transgenic Plants Overexpressing (Sense) *Arabidopsis* SNF1 Kinase

Transgenic *N. benthamiana* plants made as described above and comprising the exogenous *Arabadopsis* SNF-1 gene in sense orientation relative to the 35S promoter were tested by challenge inoculation with BCTV. Viruses were delivered to plants by the agroinoculation procedure described in Elmer et al. (1988) *Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNASs* Plant Mol. Biol. 10:225-234.

Non-transgenic *N. benthamiana* plants and plants representing three transgenic lines expressing an sense SNF1 construct (S-1, S-3, and S-5) were agroinoculated with varying doses of BCTV, beginning with the standard dose (OD600 = 1.0) followed by serial 5-fold dilutions of the standard dose. The percent of plants in the sample infected at each inoculum dose was noted and plotted versus the log5 of the dilution. The data represent the average of four independent experiments, with 16 plants for each treatment. As shown in Figure 5, the ID_{50} for BCTV on non-transgenic plants was reached with essentially undiluted inoculum in this experiment, whereas the an inoculum greater than the standard dose is needed to achieve the ID_{50} for lines S-1, S-3, and S-5.

However, it is possible to calculate by extrapolation that a dose approximately 5-fold greater (line S-5), 13-fold greater (line S-3), and more than 200-fold greater (line S1) than the standard dose is needed to achieve 50% infection of transgenic plant populations.

Thus, in the case of transgenic, sense, over-expressing SNF1 lines, it is clear that the ID_{50} is much greater than it is on non-transgenic plants (Fig. 5). That is, much more virus is required to infect a significant fraction of the transgenic plants. The plants prepared as described in the present example also had a slow growth phenotype, suggesting that use of a strong constitutive promoter such as CaMV 35 S may, in some cases, be less preferred.

While the method for preparing a transgenic plant which is more resistant to infection with a plant pathogen has been described to some degree of particularity, various adaptations and modifications can be made without departing from the scope of the invention as defined in the appended claims.

CLAIMS

What is claimed is

1. A method of transforming a plant cell which comprises

(a) introducing a DNA fragment into a plant cell, said DNA fragment comprising a polynucleotide encoding (i) a protein whose amino acid sequenc is at least 90% identical to the amino acid sequence of an SNF-1 protein kinase or (ii) a polypeptide whose amino acid sequence is at least 90% identical to the amino acid sequence of the catalytic domain of an SNF-1 protein kinase, said polynucleotide being operably linked to a promoter for expression of the protein or the polypeptide by the plant cell; and

(b) selecting a plant cell that expresses a transcript of said polynucleotide.

2. The method of claim 1 wherein the DNA fragment further comprises a polynucleotide encoding a screenable marker or a selectable marker.

3. The method of claim 1 wherein the polynucleotide encodes an amino acid sequence which is at least 95% identical to a heterologous SNF-1 kinase or a homologous SNF-1 kinase.

4. The method of claim 1 wherein the SNF-1 kinase is a plant SNF-1 kinase.

5. The method of claim 1 wherein the SNF-1 kinase is a yeast SNF-1 kanase.

6. The method of claim 1 wherein the polynucleotide encodes a polypeptide whose amino acid sequence is at least 90% identical to amino acid 1 through amino acid 350 of a plant SNF-1 kinase or to amino acid 1 through amino acid 400 of a yeast SNF-1 kinase.

8. A method of preparing a transgenic plant with enhanced resistance to a plant pathogen, said method comprising

(a) providing a transformed plant cell prepared by the method of claim 1; and

(b) generating a genetically transformed plant from said transformed plant cell, wherein said genetically transformed plant is more resistant to infection with a plant pathogen than a non-transformed plant of the same genus and species.

9. A transformed plant cell comprising a transgene, wherein the transgene is a polynucleotide which encodes an exogenous SNF-1 kinase gene or the catalytic domain of an SNF-1 kinase, said polynucleotide being operably linked to a promoter.

9. The transformed plant cell of claim 8 wherein said promoter is a constitutive promoter.

10. The transformed plant cell of claim 8 wherein the promoter is a tissue specific promoter.

11. The transformed plant cell of claim 8 wherein the promoter is an inducible promoter.

12. The transformed plant cell of claim 8 wherein the promoter is a constitutive promoter which is weaker than the 35S CaMV promoter.

13. The transformed plant cell of claim 8 wherein the transgene is stably integrated into the genome of the plant cell.

14. The transformed plant cell of claim 8 wherein a T-DNA border sequence is upstream or downstream of the transgene.

15. A transgenic plant with enhanced resistance to a plant pathogen or to abiotic stress, said transgenic plant comprising the transformed plant cell of claim 8.

16. The transgenic plant of claim 15 wherein said transgenic plant is a monocotyledonous plant.

17. The transgenic plant of claim 15 wherein said transgenic plant is a dicotyledonous plant.

18. The transgenic plant of claim 15 wherein said transgenic plant has enhanced resistance to a viral pathogen.

19. The transgenic plant of claim 15 wherein said transgenic plant has enhanced resistance to a Geminivirus.

20. A transgenic plant transformed with and expressing an exogeonous SNF-1 kinase or the catalytic domain of an SNF-1 kinase, wherein expression of said exogenous SNF-1

kinase or said catalytic domain results in enhanced resistance of said transgenic plant to a pathogen.

21. A seed derived from a transgenic plant as defined in claim 20.

22. Propagating plant material derived from a transgenic plant as defined in claim 20.

Fig. 1

DNA sequence 1539 b.p. atggatcaattca...cgtgtgatcrga linear

```
1/1                                                 31/11
atg gat cat tca tca aat aga ttt ggc aat aat gga gtg gaa tcq att tta ccg aat tac
M   D   H   S   S   N   R   F   G   N   N   G   V   E   S   I   L   P   N   Y
61/21                                               91/31
aag ctt ggt aaa act ctt gga att ggg tct ttt ggg aag gtg aaa ata gca gag cat gtt
K   L   G   K   T   L   G   I   G   S   F   G   K   V   K   I   A   E   H   V
121/41                                              151/51
gtc aca ggg cat aag gtt gct atc aaa atc ctt aat cgt cgt aag atc aag aac atg gag
V   T   G   H   K   V   A   I   K   I   L   N   R   R   K   I   K   N   M   E
181/61                                              211/71
atg gaa gag aaa gtg agg agg gag att aag att cta cgg ttg ttt atg cat cct cat att
M   E   R   K   V   R   R   E   I   K   I   L   R   L   P   M   H   P   H   I
241/81                                              271/91
att cgg cag tat gag gta ata gag acc acg agt gac att tat gtt gtg atg gag tat gtc
I   R   Q   Y   E   V   I   E   T   T   S   D   I   Y   V   V   M   R   Y   V
301/101                                             331/111
aag tct gga gag ctc ttt gat tat att gtt gag aaa ggc aga tta caa gaa gat gag gct
K   S   G   B   L   F   D   Y   I   V   E   K   G   R   L   Q   E   D   E   A
361/121                                             391/131
cgt aac ttt ttc cag cag ata ata tct ggt gta gag tac tgc cat cgt aat atg gtt gtc
R   N   F   F   Q   Q   I   I   S   G   V   E   Y   C   H   R   N   M   V   V
421/141                                             451/151
cat aga gac ctg aag oct gag aat tta cta ttg gat tcg agg tgt aat att aag att gca
H   R   D   L   K   P   E   N   L   L   L   D   S   R   C   N   I   K   I   A
481/161                                             511/171
gad ttt ggg ttg agt aat gtt atg cgg gat ggt oat ttt cta aag acg agt tgt gga agc
D   F   G   L   S   N   V   M   R   D   G   H   F   L   K   T   S   O   G   S
541/181                                             571/191
ccc aac tac gct gct ccc gag gtt ata tca ggt aaa tta tat gct gga cct gaa gta gat
P   N   Y   A   A   P   E   V   I   S   G   K   L   Y   A   G   P   Z   V   D
601/201                                             631/211
gta tgg agt tac gga gtt ata ttg tac gct cta tta tgd ggt act ctt cot ttt gat gat
V   W   S   C   G   V   U   L   Y   A   L   L   C   G   T   L   P   F   D   D
621/221                                             691/231
gaa aac att ccc aac ctt ttc aag aaa att aag ggt ggg att tac act ctt cca agt cat
E   N   I   P   N   L   F   K   K   I   K   G   G   I   Y   T   L   P   S   K
721/241                                             751/251
tta tca tct gag gct aga gac ctg atc cca agg atg ctt ata gtt gac ccg gtg aaa cga
L   S   S   E   A   R   D   L   I   P   A   M   L   I   V   D   P   V   K   A
781/261                                             811/271
atc acc att cct gag atc cgt caa cac cgt tgg ttc cag act cat ctc cct cgt tat ctt
I   T   I   P   E   I   R   Q   H   R   W   F   Q   T   H   L   P   R   Y   L
841/281                                             871/291
gct gtc tct cca ccg gat aca gta gag cag act aaa aag atc aat gag gag ata gtt caa
A   V   S   P   P   D   T   V   E   Q   T   K   K   I   N   E   E   I   V   O
901/301                                             931/311
gaa gtg gtt aac atg gga ttt gat aga aac cag gtt ttg gaa tct cta cgc aac aga aca
E   V   V   N   M   G   F   G   R   N   Q   V   L   E   S   L   R   N   R   T
961/321                                             991/331
caa aac gat gct act gtt aca tac tac ctg tta ttg gat aac cgg ttc cgt gtt cca agt
Q   N   D   A   T   V   T   Y   Y   L   L   L   D   N   R   F   R   V   P   S
1021/341                                            1051/351
ggc tat cta gaa tcc gag ttt cag gag aca aca gac agt ggc tcc aat cct atg cgc aca
G   Y   L   E   S   E   F   Q   E   T   T   D   S   G   S   N   P   M   R   T
1081/361                                            1111/371
cct gaa gcg ggc gct tca cct gta ggc cag tgg att cct gca cat gtg gat cac tac ggg
P   E   A   G   A   S   P   V   G   H   W   I   P   A   H   V   D   H   Y   G
```

Fig. 1 (con't)

```
1141/381                                        1171/391
ttg gga gca aga tca caa gtc cct gtt gat cga aaa tgg gct ctt gga ctt cag tct cat
L   G   A   R   S   Q   V   P   V   D   R   K   N   A   K   G   L   W   S   K
1201/401                                        1231/411
gcg cat cct cgt gaa atc atg aat gaa gtt ttg aaa gct ctt caa gaa ctc aat gtg tgt
A   H   P   R   E   I   M   N   E   V   L   K   A   L   Q   E   L   N   V   C
1261/421                                        1291/431
tgg aag aag att ggt cac tac aac atg aaa tgt cga tgg gtt cct ggt tta gct gat ggt
W   X   K   I   G   H   Y   N   M   K   C   R   W   V   P   G   L   A   D   G
1321/441                                        1351/451
cag aat act atg gtc aac aat cag ctg cac ttc aga gat gaa tcc agc atc att gag gat
Q   N   T   M   V   N   N   Q   L   H   F   R   D   E   S   S   I   I   E   D
1381/461                                        1411/471
gac tgt gcc atg act tca ccc act gtc atc aaa ttt gaa ctt cag cta tac aaa gcc cgg
D   C   A   M   T   S   P   T   V   I   K   F   E   L   Q   L   Y   K   A   R
1441/481                                        1471/491
gaa gag aag tac ttg ctg gat ata cag aga gtt aac ggt ccg cag ttt ctc ttc ttg gat
E   E   K   Y   L   L   D   I   Q   R   V   N   G   P   Q   P   L   P   L   D
1501/501                                        1531/511
cta tgc gcc gcc ttt ctt aca gag ctt cgt gtg atc tga
L   C   A   A   F   L   T   E   L   R   V   I   *
```

Fig. 2

Alignment Report of Untitled, using Clustal method with PAM250 residue weight table.
Friday, August 6, 1999 4:07 PM


```
     M D X S X N - - - - - - - - - - - - - - - - - - - - - - X X G X X G V E S X L X   Majority
                       10                  20                  30                  40
 1   M D H S S N - - - - - - - - - - - - - - - - - - - - - - R F G N N G V E S I L P   Arabidopsis SNF1
 1   M D G S T V - - - - - - - - - - - - - - - - - - - - - - Q - G G S S V E S F L R   Tobacco SNF1 (NPK5)
 1   M S S N N N T N T A P A N A N S S H H H H H H H H H H H H H G H G G S N S T L N   Yeast SNF1

     N - - - - - - - - - - - - - Y K L G X T L G I G S F G K V K I A E H T X T G H K   Majority
                       50                  60                  70                  80
19   N - - - - - - - - - - - - - Y K L G K T L G I G S F G K V K I A E H V V T G H K   Arabidopsis SNF1
18   N - - - - - - - - - - - - - Y K L G K T L G I G S F G K V K I A E H T L T G H K   Tobacco SNF1 (NPK5)
41   N P K S S L A D G A H I G N Y Q I V K T L G E G S F G K V K L A Y H T T T G Q K   Yeast SNF1

     V A X K I L N R R K I K N M E M E E K V R R E I K I L R L F M H P H I I R L Y E   Majority
                       90                 100                 110                 120
46   V A I K I L N R R K I K N M E M E E K V R R E I K I L R L R M H P H I I R Q Y E   Arabidopsis SNF1
45   V A V K I L N R R K I K N M E M E E K V R R E I K I L R L F M H P H I I R L Y E   Tobacco SNF1 (NPK5)
81   V A L K I I N K K V L A K S D M Q G R I E R E I S Y L R L L R H P H I I K L Y D   Yeast SNF1

     V I E T X S D I Y V V M E Y V K S G E L F D Y I V E K G R L Q E D E A R X F F Q   Majority
                      130                 140                 150                 160
86   V I E T T S D I Y V V M E Y V K S G E L F D Y I V E K G R L Q E D E A R N F F Q   Arabidopsis SNF1
85   V V E T P S D I Y V V M E Y V K S G E L F D Y I V E K G R L Q E D E A R K F F Q   Tobacco SNF1 (NPK5)
121  V I K S K D E I I M V I E Y A - G N E L P D Y I V Q R D K M S E Q E A R R F F Q   Yeast SNF1

     Q I I S G V E Y C H R N M V V H R D L K P E N L L L D S X X N V K I A D F G L S   Majority
                      170                 180                 190                 200
120  Q I I S G V E Y C H R N M V V H R D L K P E N L L L D S R C N I K I A D F G L S   Arabidopsis SNF1
125  Q I I S G V E Y C H R N M V V H R D L K P E N L L L D S K W N V K I A D F G L S   Tobacco SNF1 (NPK5)
160  Q I I S A V E Y C H R H K I V H R D L K P E N L L L D E H L N V K I A D F G L S   Yeast SNF1

     N I M R D G H F L K T S C G S P N Y A A P E V I S G K L Y A G P E V D V W S C G   Majority
                      210                 220                 230                 240
166  N V M R D G H F L K T S C G S P N Y A A P E V I S G K L Y A G P E V D V W S C G   Arabidopsis SNF1
165  N I M R D G H F L K T S C G S P N Y A A P E V I S G K L Y A G P E V D V W S C G   Tobacco SNF1 (NPK5)
200  N I M T D G N F L K T S C G S P N Y A A P E V I S G K L Y A G P E V D V W S C G   Yeast SNF
```

Fig. 2 (con't)

Alignment Report of Untitled, using Clustal method with PAM250 residue weight table.

Friday, August 6, 1999 4:07 PM

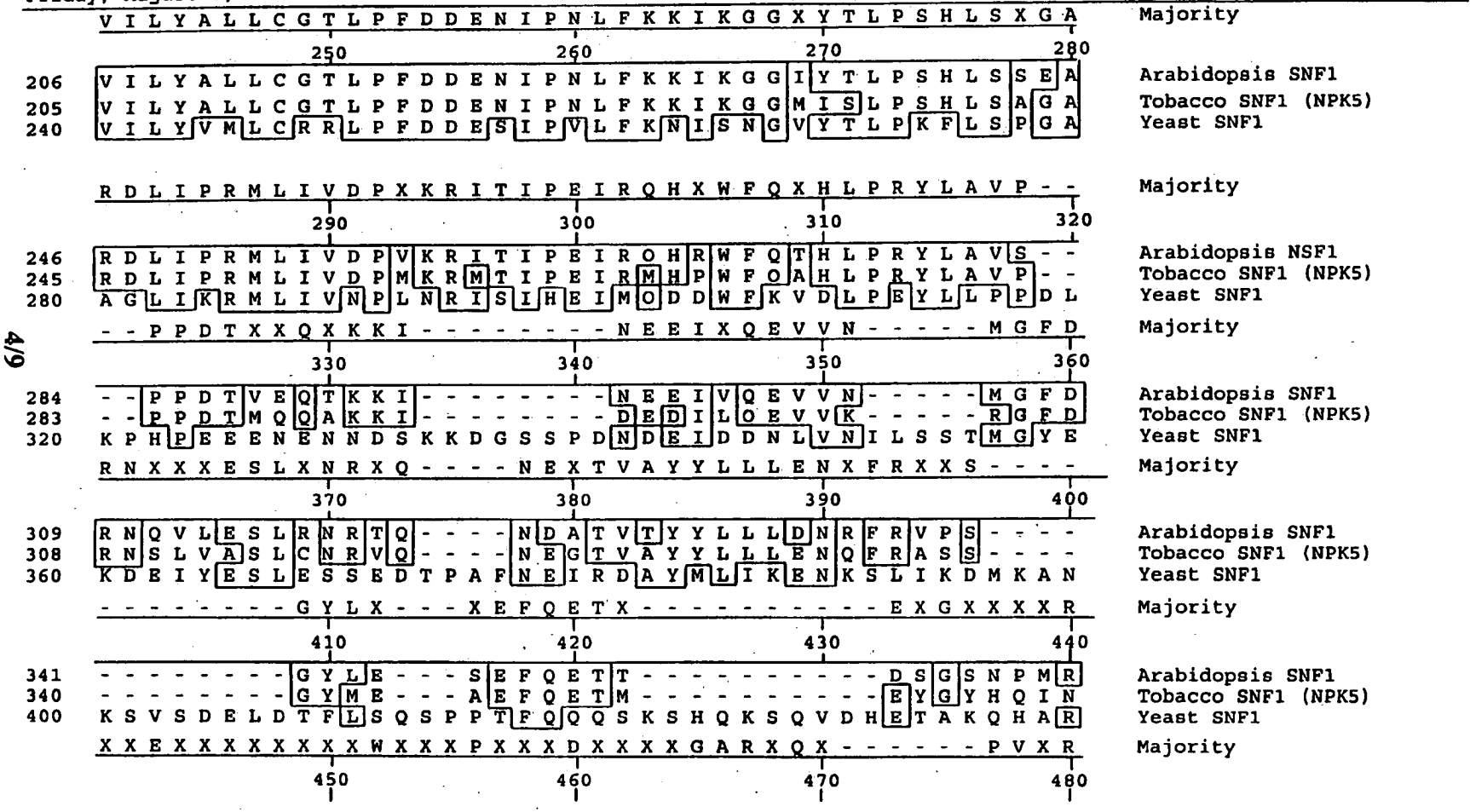


Fig. 2 (con't)

Alignment Report of Untitled, using Clustal method with PAM250 residue weight table. Page 3
Friday, August 6, 1999 4:07 PM

```
360 TPEAGASPVGHW--IPAHVDHYGLGARSQV------PVDR  Arabidopsis SNF1
359 SSEV---LLPCWQHLPGIMDFQQVGAR-QF------PVER  Tobacco SNF1 (NPK5)
440 RMASAITOORTYHOSPFMDOYKEEDSTVSILPTSLPQIHR  Yeast SNF1
    ----------------------KWALGLQSRAHPREIN  Majority

                490       500       510       520
392 ----------------------KWALGLQSHAHPREIM  Arabidopsis SNF1
389 ----------------------KWALGLOSRAHPREIM  Tobacco SNF1 (NPK5)
480 ANMLAQGSPAASKISPLVTKKSKTRWHFGIRSRSYPLDVM  Yeast SNF1

    XEVLKALQXLNVXWKKIGXYNM---KCRW---VPGXXXXX  Majority

                530       540       550       560
408 NEVLKALQELNVCWKKIGHYNM---KCRW---VPGLADGQ  Arabidopsis SNF1
405 TEVLKALOGLNVRWKKIGPYNM---KCQW---VPGVPGHH  Tobacco SNF1 (NPK5)
520 GEIYIALKNLGAEWAKPSEEDLWTIKLRWKYDIGNKTNTN  Yeast SNF1

    EXMXN-NXXHIOXFXDESXXIEXX-XXTXPXXVKFEXOLY  Majority

                570       580       590       600
442 NTMVN-NQLH---FRDESSIIEDDCAMTSPTVIKFELQLY  Arabidopsis SNF1
439 EGMSN-NSIHIQFFGDDSTVIENG-GVTIPNAVKFEVOLY  Tobacco SNF1 (NPK5)
560 EKIPDLMKMVIOLFQIE----------TNNYLVDFK---F  Yeast SNF1

    KXREEKY---------LLDXQRVXGPQFLFLDLCAAFLXEL  Majority

                610       620       630       640
478 KAREEKY---------LLDIQRVNGPQFLFLDLCAAFLTEL  Arabidopsis SNF1
477 KTREEKY---------LLDLQRVQGPOFLFLDLCAAFLAQL  Tobacco SNF1 (NPK5)
587 DGWESSYGDDTTVSNISEDEMSTFSAYPFLHLTTKLIMEL  Yeast SNF1

    RVXE----  Majority
```

Fig. 2 (con't)

Alignment Report of Untitled, using Clustal method with PAM250 residue weight table. Page 4
Friday, August 6, 1999 4:07 PM

510	R V I Z	Arabidopsis SNF1
509	R V L Z	Tobacco SNF1 (NPK5)
627	A V N S Q S N Z	Yeast SNF1

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Fig. 3

Mean latent period following BCTV Inoculation of transgenic antisense SNF1 plants

	N. benthamiana line			
Virus	**Non-transgenic**	**AS-4**	**AS-5**	**AS-12**
BCTV	**21.17+/-1.35 (6/14)**	**16.4+/-1.29 (14/15)**	**14.7+/-0.87 (10/16)**	**14.63+/-0.84 (13/14)**

Fig. 4

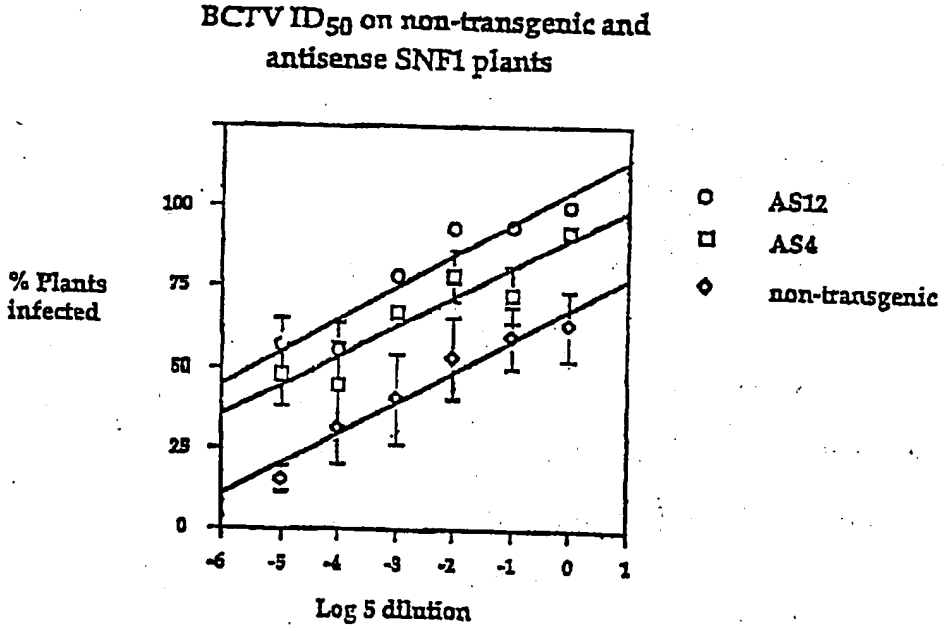
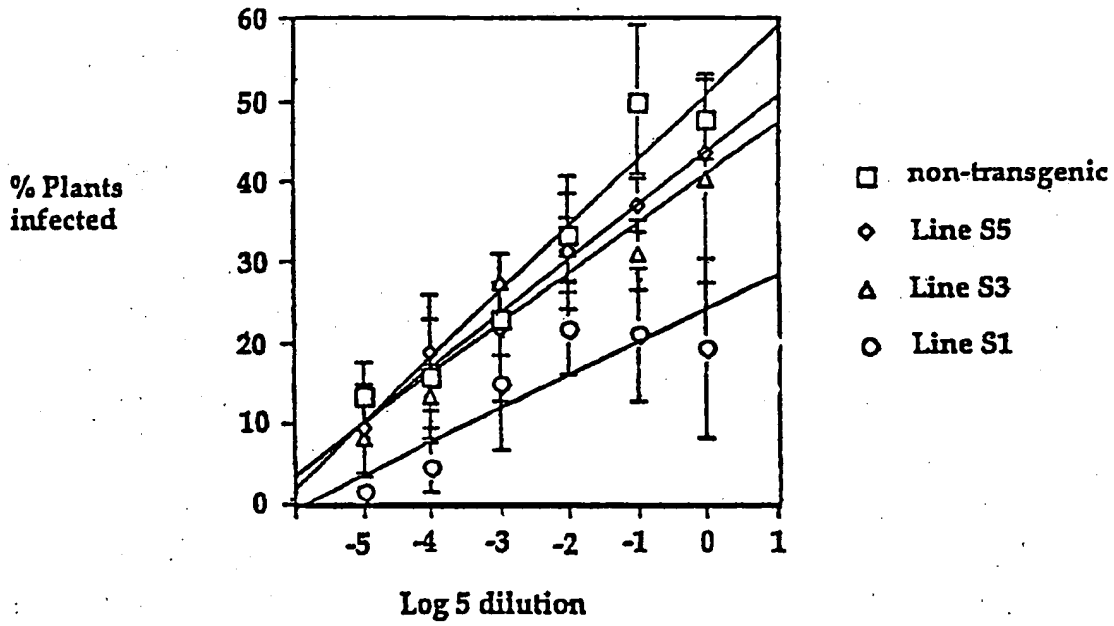

BCTV ID50 on non-transgenic and antisense SNF1 plants
% Plants infected
100
75
50
25
0
-6
-5
-4
-3
-2
-1
0
1
Log 5 dilution
AS12
AS4
non-transgenic

Fig. 5

BCTV ID_{50} on non-transgenic and sense (over-expressing) SNF1 plants



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/21624

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/05, 15/09, 15/29, 15/31, 15/82; A01H 5/00, 5/10

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 468, 419; 536/23.1, 23.6, 23.7; 800/278, 279, 287, 288, 295, 298, 301

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS, WEST2.0

terms: SNF-1 kinase, Arabidopsis, pathogen-resistant, transformed plant cell, serine/threonine, viral resistant plants, geminivirus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BHALERAO et al. Regulatory Interaction of PRL1 WD Protein with Arabidopsis SNF1-like Protein Kinases. Proc. Natl. Acad. Sci. USA. April 1999, Vol. 96, pages 5322-5327, see entire document.	1-22
Y	LE GUEN et al. Structure and Expression of a Gene from Arabidopsis thaliana Encoding a Protein Related to SNF1 Protein Kinase. Gene. 1992, Vol. 120, pages 249-254, see entire document.	1-22
Y	SUGDEN et al. Two SNF1-Related Protein Kinases from Spinach Leaf Phosphorylate and Inactive 3-Hydroxy-3-Methyglutaryl-Coenzyme A Reductase, Nitrate Reductase, and Sucrose Phosphate Synthetase in Vitro. Plant Physiology. May 1999, Vol. 120, pages 257-274, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 03 NOVEMBER 2000	Date of mailing of the international search report 28 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MEDINA A. IBRAHIM Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/21624

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 468, 419; 536/23.1, 23.6, 23.7; 800/278, 279, 287, 288, 295, 298, 301